PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Q. Nguyen
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For	:	METHOD OF INDUCING NEURONAL PRODUCTION IN THE BRAIN AND SPINAL CORD	

SECOND DECLARATION OF STEVEN A. GOLDMAN UNDER 37 C.F.R. §1.132

Mail Stop: RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor and Chief, Division of Cell and Gene Therapy, Glenn-Zutes Chair in Biology of the Aging Brain, Department of Neurology, University of Rochester Medical Center, Rochester, New York.

3. I am a named inventor of the above patent application.

Striatal Neuronal Addition in Both Wild-type and R6/2 Mice

4. To test the feasibility of using an adenovirus containing BDNF ("AdBDNF") or noggin ("AdNoggin") to induce striatal neurogenesis to treat Huntington's Disease, students in my laboratory working under my direction injected AdBDNF and

AdNoggin intraventricularly into Huntington mutant R6/2 mice and into normal wild-type mice. The results are described below.

5. Replication-incompetent AdBDNF and AdNull were constructed and raised as described (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001)). In brief, an adenoviral vector bearing BDNF under the control of the cytomegalovirus (CMV) promoter was constructed, placed upstream to the gene encoding humanized green fluorescent protein (hGFP), with an intervening internal ribosomal entry site (IRES). Using the same techniques, a ΔE1 type 5 adenovirus was made to encode, under CMV control, human noggin$^{\Delta B2}$, from which the B2 heparin binding domain had been deleted, yielding AdNoggin$^{\Delta B2}$ (Paine-Saunders et al., "Heparan Sulfate Proteoglycans Retain Noggin at the Cell Surface: A Potential Mechanism for Shaping BMP gradients," *J Biol Chem* (2001)).

6. A cohort of 4 week-old R6/2 (n = 18) and WT (n = 18) mice received bilateral 1.5 μl intraventricular injections of either AdBDNF/AdNoggin (n = 4 each), AdBDNF (n = 4 each), AdNull (n = 7 each), or saline (n = 3 each) (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001)), stereotaxically delivered to the following coordinates: from Bregma, AP –0.5 mm, ML ±0.7 mm; from dura, DV –2.0 mm. For AdBDNF/AdNoggin group, AdBDNF, and AdNoggin$^{\Delta B2}$ were both brought to a titer of $2.5*10^{10}$ pfu/ml within the same solution, and this cocktail were injected. The mice were then injected daily for 30 days with the mitotic marker BrdU (100 mg/kg, i.p.). They were then sacrificed 2 weeks after the last BrdU injection, at 10 weeks of age. In addition, R6/2 (n=4) and WT (n=4) mice were injected daily for 4 weeks with BrdU (100 mg/kg, i.p.), over the period spanning 9-12 wks of age, then sacrificed and examined at 13 weeks, so as to investigate endogenous neurogenesis in HD striata.

7. A cohort of 4 animals received intraventricular AdBDNF/AdNoggin (1 R6/2 and 3 wild-type littermates) were injected with AdBDNF±AdNoggin, followed by daily injections of BrdU for 3 weeks. Three weeks after the last BrdU injection, they were injected with 1 μl of 1% Fluorogold (Biotium, Hayward, CA) bilaterally into the globus pallidus (from Bregma: AP –0.8 mm, ML ±2.25 mm; DV –3.25 mm (Paxinos et al., "The Rat Brain in Stereotaxic Coordinates," 2 ed (Orlando, FL, Academic) (1986)). The injected animals were

sacrificed 10 days later, perfused with 2% paraformaldehyde, and their brains cryosectioned and stained for BrdU, followed by confocal identification of $BrdU^+$/$Fluorogold^+$ striatal cells.

8. The animals were sacrificed, perfusion fixed, and their brains removed on day 32 after viral injection. Fixation was accomplished with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, followed by serial immersion in 6% and 30% sucrose in PB. The brains were cryosectioned as 15 μm sagittal sections, then stained for BrdU and neuronal markers as previously described (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001); Roy et al., "Identification, Isolation, and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter," *J Neurosci* 19:9986-9995 (1999); Roy et al., "In Vitro Neurogenesis by Progenitor Cells Isolated from the Adult Human Hippocampus," *Nature Medicine* 6:271-277 (2000)). Individual sections were first stained for one of the following neuronal markers: 1) βIII-tubulin, using MAb TuJ1 at 1:400 (Promega, Madison, WI); 2) DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) (Dr. H. Hemmings); 3) GAD67 (glutamic acid decarboxylase 67) (Chemicon, Temecula, CA); or 4) NeuN (Chemicon). The sections were then washed and denatured in 2N HCl at 37°C for 30 min, then stained for BrdU using rat anti-BrdU antibody at 1:200 (Serotec, Raleigh, NC), followed by a goat anti-rat Alexa 488 secondary antibody (1:400; Molecular Probes, Inc., Eugene, OR).

9. In sections double-stained for BrdU and either βIII-tubulin, DARPP-32, GAD67 or NeuN, single striatal BrdU+ cells were randomly selected for confocal imaging. Using an Olympus Fluoview confocal microscope, images were acquired using an argon-krypton laser, and were analyzed as previously described (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001)). Briefly, the images of every cell double-immunostained with BrdU and a neuronal marker were observed orthogonally in both the vertical and horizontal plane. Each potentially double-labeled cell underwent independent review by 2 observers. Only when both observers deemed a cell as double-labeled with central BrdU immunoreactivity surrounded by complete neuronal staining from all observation angles in every serial optical section, and in each merged and rotated composite, were the cells scored as double-labeled new neurons.

10. Striatal BrdU+ cells counts were done on six 15μm sagittal sections per animal; every 16th section was analyzed at 240 μm intervals, as previously described (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001)). The striatal region sampled began with the first appearance of striatal fascicles. In each striatum, total BrdU+ nuclei and βIII-tubulin+ neurons were counted at 20x magnification; these results were converted into BrdU+ cells/mm^3 or neurons/mm^3 after determining the striatal surface area using BioQuant image analysis software, with which the net volume of each striatum was estimated. The number of striatal BrdU+/βIII-tubulin+ cells/mm^3 in a given section was then determined by multiplying the percentage of BrdU+ cells that co-expressed βIII-tubulin in confocal-verified sample fields, by the total number of BrdU+cells/mm^3. Comparisons of the number of BrdU+/βIII-tubulin+ cells/mm^3 in AdBDNF/AdNoggin, AdBDNF-, AdNull-, and saline-injected animals were performed using ANOVA and post hoc Bonferroni t-tests. Statistical analyses were performed using GB-Stat or SPSS.

11. AdBDNF induced striatial neuronal addition in both wild-type and R6/2 mice. A total of 36 mice, 18 R6/2, and 18 controls, were used to assess the ability of BDNF ± noggin treatment to elicit striatal neurogenesis in R6/2 mice, relative to their WT controls. At 4 weeks of age, matched groups of R6/2 (n = 18) and WT (n = 18) mice received bilateral 1.5 μl intraventricular injections of either: 1) AdBDNF/AdNoggin (n = 4 each, for both R6/2 and WT mice); 2) AdBDNF (n = 4 each); 3) AdNull (n = 7 each); or 4) saline (n = 3 each). All mice were treated with BrdU once daily for 4 weeks thereafter, and then sacrificed at 10 wks of age. Their brains were sectioned and their striata immunolabeled for BrdU together with either NeuN or βIII-tubulin, so as to identify newly generated neurons, using confocal validation as described (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001) and Kuhn et al., "Epidermal Growth Factor and Fibroblast Growth Factor-2 Have Different Effects on Neural Progenitors in the Adult Rat Brain," *J Neurosci* 17:5820-5829 (1997)). Among the R6/2 mice, AdBDNF-treated animals exhibited 135.0 ± 34.1 BrdU+/βIII-tubulin+ cells/mm^3, significantly more than their counterparts given AdNull (20.1 ± 6.5) or saline (5.1 ± 7.8) ($p<0.05$ by ANOVA followed by post hoc Bonferroni t-test). Similarly, wild-type mice injected solely with AdBDNF exhibited 84.3 ± 30.5 BrdU+/βIII-tubulin+ cells/mm^3,

significantly more than mice given AdNull (13.1 ± 9.7) or saline (4.2 ± 3.0) ($p<0.01$). Thus, AdBDNF treatment elicited substantial neuronal addition to the neostriata of both R6/2 transgenic and wild-type mice. In both, the neuronal phenotype of AdBDNF-induced BrdU+/βIII-tubulin+ cells was confirmed by immunolabeling for NeuN. In neither the R6/2 mice nor wild-types was any evidence of striatal neurogenesis observed in saline-treated controls, at least among mice sacrificed at 10 weeks of age.

12. AdBDNF/AdNoggin cooperatively induced striatal neuronal recruitment. It was next postulated that by virtue of its suppression of gliogenesis (Chmielnicki et al., "Adenoviral Co-Delivery of BDNF and Noggin Cooperate to Induce New Medium Spiny Neurons from Resident Progenitor Cells in the Adult Forebrain," *Journal of Neuroscience*, in press (3/04) (2004)), noggin overexpression might increase the pool of ventricular zone progenitors responsive to neuronal instruction by BDNF. It was found that the concurrent use of AdBDNF and AdNoggin, by effectively providing a permissive and instructive environment for striatal neurogenesis, greatly enhanced neuronal recruitment to the striatum. WT mice treated with AdBDNF/AdNoggin exhibited 219.9 ± 19.4 BrdU+/βIII-tubulin+ cells/mm^3, significantly more than AdBDNF-, AdNull- or saline injected WT mice ($p<0.01$ by ANOVA, with post hoc Bonferroni t-test). Similarly, the neostriata of AdBDNF/AdNoggin-treated R6/2 mice harbored 277.0 ± 52.7 BrdU+/βIII-tubulin+ cells/mm^3, significantly more than observed in R6/2 mice given AdBDNF, AdNull, or saline (each $p<0.05$ by ANOVA with post hoc Bonferroni t-tests). Thus, in both WT and R6/2 mice, BDNF and noggin acted cooperatively to induce striatal neuronal recruitment. Importantly, AdBDNF/AdNoggin treatment elicited as strong a neurogenic response in R6/2 mice as in WT mice, suggesting that neither the mutant huntingtin phenotype, nor any antecedent compensatory progenitor response, had depleted or exhausted the progenitor pool in R6/2 mice.

13. New striatal neurons were recruited as medium spiny neurons. It had been previously noted in rats that AdBDNF-induced striatal neurons expressed markers characteristic of GABAergic medium spiny neurons, such as calbindin, GAD67, GABA, and DARRP-32 (Benraiss et al., "Adenoviral Brain-Derived Neurotrophic Factor Induces both Neostriatal and Olfactory Neuronal Recruitment from Endogenous Progenitor Cells in the Adult Forebrain," *J Neurosci* 21:6718-6731 (2001)). To determine if newly recruited neurons in the R6/2 striatum likewise differentiated as MSNs, sections of BrdU-tagged R6/2 striata were immunolabeled for both BrdU and DARPP-32, the most definitive marker for the

GABAergic medium spiny neuron phenotype within the striatum (Ivkovic et al., "Expression of the Striatal DARPP-32/ARPP-21 Phenotype in GABAergic Neurons Requires Neurotrophins In Vivo and In Vitro," *J Neurosci* 19:5409-5419 (1999) and Ivkovic et al., "Brain-Derived Neurotrophic Factor Regulates Maturation of the DARPP-32 Phenotype in Striatal Medium Spiny Neurons: Studies In Vivo and In Vitro," *Neuroscience* 79:509-516 (1997)). The resultant counts were compared to those obtained from R6/2 mice injected with either AdBDNF alone or AdNull. In addition, counts were obtained from matched wild-type controls similarly injected with either AdNull, AdBDNF, or AdBDNF/AdNoggin. It was found that in R6/2 mice injected with AdBDNF/AdNoggin, 10.1 ± 4.6% of the imaged BrdU+ cells, or 167.4 ± 52.5 cells/mm^3, were DARPP-32+. In contrast, only 69.8 ± 5.8 DARPP-32+/BrdU+ striatal neurons were noted in R6/2 mice treated with AdBDNF alone, and those injected with AdNull exhibited no DARPP-32+/BrdU+ striatal neurons at all, consistent with their lack of spontaneous striatal neurogenesis at this age. The difference between AdBDNDF/AdNoggin- and AdNull-treated R6/2 mice in their number of newly recruited striatal DARPP32+ neurons, like that demonstrated previously in their number of overall new neurons, proved significant ($F=11.3$, $p<0.05$; 2-way analysis of variance with post hoc Boneferroni t-tests). Wild-type mice exhibited the same effect, with AdBDNF/AdNoggin yielding a significant recruitment of new DARPP-32+/BrdU+ neurons to the normal neostriatum. Indeed, the specific induction of DARPP32+ medium spiny neurons by AdBDNF/AdNoggin proved greater in R6/2 mice than their wild-type controls; induced medium spiny neurogenesis thus proved even more robust in the R6/2 mouse as in normal wild-type adults.

14. Newly generated striatal neurons developed projections to the globus pallidus. It was next asked whether the new neurons of the caudate-putamen extended processes to their normal developmental target, the globus pallidus. To address this question, the retrograde tracer Fluorogold was injected into the globus pallidus of mice given AdBDNF/Noggin or AdBDNF 6 weeks earlier, who had been injected daily with BrdU for 3 weeks after virus administration. Ten days after Fluorogold injection (7-8 weeks after initial virus administration), the mice were sacrificed. Their striata were assessed for the incidence of BrdU+/Fluorogold+ cells, which were defined thereby as newly generated pallidal projection neurons. It was found that 14.2 ± 1.3 BrdU+/Fluorogold+ striatal neurons/mm^3 in AdBDNF-injected mice, and 20.0 ± 9.6 neurons/mm^3 in AdBDNF/AdNoggin-treated animals, projected to the globus pallidus. Since the globus pallidus is the major target of

medium spiny axons, these results suggest that newly-generated medium spiny neurons can project axons to their appropriate postsynaptic targets.

AdBDNF/AdNoggin Delayed Functional Deterioration

15. To demonstrate that the use of AdBDNF and AdNoggin in combination delayed functional deterioration, motor coordination and balance were measured using rotarod analysis (Andreassen et al., "Creatine Increase Survival and Delays Motor Symptoms in a Transgenic Animal Model of Huntington's Disease," *Neurobiol Dis* 8:479-491 (2001)). Both AdBDNF/AdNoggin (n=6) and AdNull (n=6) treated R6/2 mice were assessed by rotorod, as were their wild-type controls, beginning at 3 weeks of age. The mice were trained three times daily for two consecutive days on a rotarod (Columbus Instruments, Columbus, OH), at a constant speed of 12 rpm; they were subsequently tested weekly at same speed. At each weekly test, each mouse was given three trials on the rod, and their latencies to fall measured. Maximum latency was defined as 300 sec., at which the individual test was terminated and scored as 300 sec; for every 3-trial test, the best result, i.e., the longest time spent on the rod without falling, was recorded. All mice were tested from the day before stereotaxic surgery, at 4 weeks of age, until they died or they were unable to maintain their body posture. Rotarod scores of < 60 sec were considered neurologically abnormal (Laforet et al., "Changes in Cortical and Striatal Neurons Predict Behavioral and Electrophysiological Abnormalities in a Transgenic Murine Model of Huntington's Disease," *J Neurosci* 21:9112-9123 (2001)). Within the 0-60 sec range, a 7-point score was used to describe motor coordination, in which each 10 sec of sustained rotorod performance was equivalent to 1 point; with this scoring system, the rotorod performance of both AdBDNF/AdNoggin and AdNull mice was quantitatively graded. Statistical comparisons of the average duration of rotarod performance as a function of age were performed by 2-way ANOVA. The latency to onset of motor dysfunction was separately assessed, also by 2-way ANOVA comparing treatments as a function of age.

16. It was found that the AdBDNF/AdNoggin-treated mice exhibited a significant deceleration in motor deterioration, relative to both their saline and AdNull-treated R6/2 controls. When the latency to fall off the rotorod (y) was plotted as a function of post-operative survival (m), curves were generated for AdBDNF/AdNoggin- and AdNull-injected mice that appeared to diverge at approximately 5 weeks after treatment. Simple regression analysis with curve-fitting according to y=mx+b revealed that whereas the motor

performance of AdBDNF/AdNull-treated animals could be described by the line y = -43.9x + 302.5, that of their AdNull-treated controls was described by y = -70.8 + 319.5. Mann Whitney analysis of these regressions revealed that the rate of deterioration of motor performance, as reflected in the regression slopes, was significantly greater in AdNull animals (m=-70.8 ± 7.9) than in their AdBDNF/AdNoggin-treated counterparts (-43.9 ± 3.9; p=0.007). Because of their slower rate of deterioration, the AdBDNF/AdNoggin-treated R6/2 mice exhibited a delayed onset in their motor abnormality. Using a 7-point scoring of motor coordination, in which each 10 sec of sustained rotorod performance equals 1 point (yielding a standard score of 7 for performance of >60 sec), the rotorod performance of both AdBDNF/AdNoggin and AdNull mice was quantitatively graded. It was found that whereas the AdBDNF/AdNoggin treated R6/2 mice were able to remain on the rotorod for at least one minute, and hence to maintain a maximal score of 7, until 9.5 ± 0.8 wks of age, AdNull treated mice failed to maintain this capability as of 7.3 ± 0.5 weeks (p= 0.07 by Student t-test). As a result, at 5 weeks post-operatively, AdBDNF/AdNoggin-treated R6/2 mice were able to remain on the 12 rpm rotarod for an average of 57.2 ± 18.9 sec, while their AdNull treated controls fell off in an average of 18.0 ± 1.4 sec. Similarly, at 6 weeks, treated R6/2 mice maintained rotorod performance for 51.2 ± 39 sec, while the AdNull fell off the rotarod in an average of only 7.5 ± 2.1 sec. When the treated and null control mice were compared to one another, after normalization to their standard scores at each time point, the differences between the AdBDNF/Noggin and AdNull groups were seen to be significant at 5 and 6 weeks (p=0.01 and p=0.05, respectively). Statistical analysis of motor performance at later time points became impractical, however, because the untreated animals exhibited less survival than their AdBDNF/noggin-treated counterparts. Together, these results suggest that BDNF/noggin-associated neuronal addition to the R6/2 striatum slows the deterioration of motor performance in R6/2 huntingtin mice.

17. These results indicate that the concurrent overexpression of BDNF and noggin may be used to induce neuronal recruitment from endogenous progenitor cells in the R6/2 huntingtin mutant neostriatum. When these mice were assessed at 10 weeks of age, BDNF overexpression beginning at 4 weeks was found to have induced the addition of at least 135 new neurons/mm^3. Although significant, this represents <1% of the striatal neuronal population. On this basis, BDNF was co-expressed together with noggin to suppress non-neurogenic pathways of subependymal cell differentiation, and thereby increase the pool of progenitor cells potentially responsive to BDNF. By this means, >400 neurons/mm^3 were added to the AdBDNF/AdNoggin-treated R6/2 striatum within 6 weeks of viral injection.

Rotarod testing revealed that concurrent AdBDNF/AdNoggin treatment delayed the onset of motor deterioration in R6/2 mice, and slowed the progression of their motor deterioration, relative to AdNull- and saline-treated controls. These results confirmed that induced neurogenesis may be associated with a delay of symptom progression in a prototypic mouse model of Huntington's Disease. These results suggest that induced neurogenesis from resident progenitor cells may comprise a feasible strategy for therapeutic neuronal replacement in Huntington's Disease, and more generally, as a means of reconstituting lost multinuclear circuits in the diseased adult forebrain.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 3/9/04

Steven A. Goldman, M.D., Ph.D.